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REMARKS

Applicants have amended claims 13, 26, 32 and 33 to refer to specific human nucleic acid sequences. Support for the amendment can be found throughout the specification, for example at pages 4 and 52-104. Accordingly, the amendment does not introduce new matter and its entry is respectfully requested.

To conform with this amendment, claims 15 and 16 have been cancelled.

Applicants have amended the independent claims 13 and 33 to make explicit that the alterations being detected are functional alterations. Support for the amendment can be found throughout the specification, for example, at page 5, lines 16-29, and page 16, lines 7-9. The dependent claims have been amended to conform with these amendments.

Applicants have amended claim 20 to a preferred embodiment.

Applicants have cancelled claims 34, and 36-38.

Applicants have added new claims 39 and 40 to claim preferred embodiments. Support for the new claims can be found throughout the specification, for example, at page 3, lines 27-32, and page 16 lines 15-20.

Accordingly, no new matter has been introduced by the amendments and their entry is respectfully requested.

Claims 13-26 and 28-37 were rejected under 35 U.S.C. §112, first paragraph.

Applicants submit that the rejection be withdrawn for the following reasons.

The Examiner rejected the claims directed to detection of alterations contending that any and all alterations are encompassed by the claim. Applicants respectfully submit that the specification clearly defines that the "alteration is defined as a mammalian MSH5 nucleic acid sequence that **cannot replace an endogenous** nucleotide sequence in a normal host cell **without having a detectable adverse effects** on the host cell" (page 16, lines 7-9, emphasis added). In order to expedite prosecution, Applicants have amended the claims to explicitly state that the alterations are functional alterations such as discussed, for example at page 16, first paragraph. The functional alterations cause problems, for example defective meiotic segregation, infertility

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and cancer being not responsive to treatment with alkylating agents as described in the specification. Applicants respectfully submit that a skilled artisan can easily determine from the type of a mutation, using routine techniques, whether the mutation affects the function of the gene product or not, such as discussed at page 16 of the specification.

The Examiner also rejected the claims due to their breath in covering the genus of nucleic acids encoding MSH5 citing lack of the number of examples of representative species in the specification. To expedite prosecution, Applicants have further amended the claims to the human MSH5 gene of SEQ ID NO.: 1. The specification describes these sequences in detail at pages 4 and 52-104.

Accordingly, Applicants submit that the specification clearly defines the detailed structural requirements for the method for detecting alterations in the defined nucleic acid sequence. Applicants further taught that these mutations will lead to disease states. Moreover, later published research provides corroboration for the claimed embodiments. For example, these papers confirm that alterations in human MSH5 gene are associated in the diseases described in the specification, including infertility and cancer. (See, e.g., Exhibits A and B). For example, Bawa, Gene 315:177-182 (2003) confirms that such alterations result in the cancers being tolerant to alkylating agents. Thus, claim 40 is directed to a form of personalized medicine – whether or not a treatment plan will be useful or not - as taught.

Accordingly, Applicants respectfully submit that the rejection be withdrawn.

Claims 13-37 were rejected under 35 U.S.C. §112, second paragraph. The Examiner argued that the claims as written encompass all possible polymorphisms in the SEQ ID NO:1.

Applicants respectfully disagree, and submit that the rejection be withdrawn for the following reasons. The amendments have obviated this rejection.

As discussed, *supra*, the specification states that "alteration is defined as a mammalian MSH5 nucleic acid sequence that cannot replace an endogenous nucleotide sequence in a normal host cell without having a detectable adverse effects on the host cell" (page 16, lines 7-9). Applicants submit that anyone skilled in the art having read the specification would know how to determine that the alterations coming within the scope of the claim are the ones that deleteriously affect the function of the gene product and not all possible alterations.

For example, following Applicants teachings, any skilled person in the art would have been able to use this method for a wide range of activities as taught such as for personalized cancer treatment. One would have been able to select a treatment plan for a cancer patient by determining whether or not an alkylating agent would be useful.

In light of the claim amendments and arguments as shown, *supra*, Applicants respectfully request that the rejection be withdrawn.

In view of the amended claims and the above, applicants respectfully submit that all claims are in condition for allowance. Early and favorable action is earnestly solicited. The Examiner is encouraged to contact the undersigned should there be additional questions regarding the claims.

Should additional fees be required in connection with this submission, the Commissioner is herewith authorized to charge Nixon Peabody Deposit Account No. 19-2380 for any such fees.

Respectfully submitted,

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EXHIBIT A

Animal models of genetic causes of male infertility

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I. **Keywords: animal models; genes; genetics; knockout; male infertility**

II. Introduction

In the past 10 years, more than 100 different genetically engineered mice have been developed with an abnormality in spermatogenesis. More than half of these male infertility animal models have been reported in the past three years alone. Spermatogenic defects in these models vary considerably, ranging from the inappropriate migration of primordial germ cells to the inability of mature spermatozoa to bind the zona pellucida. Many of these genetically engineered mice have been reviewed previously [1-4]. Rather than discussing all of the genes that have been connected with male infertility, the purpose of this review will be highlighting the genes that seem to have the greatest potential for being clinically relevant, based on a main phenotype of male infertility in the knockout mouse model.

Our laboratory is actively evaluating infertility candidate genes in infertile humans. Because of our interests it is important to identify genes for which the gene structure, including exons, introns, and their boundaries can be established. Therefore, each time a candidate murine gene was identified, a literature search was conducted and the NCBI databases searched to determine if a human homologue had been reported or published. In several cases there was no information currently available on the nucleotide or amino acid sequence in humans. If an amino acid sequence was available, it was used to conduct a translated BLAST search of the HTGS (high throughput genomic sequences) database of the human genome. If the structure of the gene in humans could be derived from the BLAST analysis or a published account it was included in this review. If the structure could not be determined, it was excluded.

Male Infertility candidate genes

Genes can be divided into many different groups based on the function or characteristics of the mRNAs or proteins that they produce. Examples include transcription regulators, growth factors, apoptosis regulators, enzymes, hormones, and receptors. For the purposes of this review, the genes are grouped into categories related to the defect produced in spermatogenesis when they are disrupted, or the physical location of the proteins the genes code for in the developing spermatozoa.

Genes related to defects in Meiosis

Meiotic cell division is unique to the production of mature gametes. Meiosis proceeds differently from mitotic cell division in several ways, most notably in how the chromosomes pair and divide. Many of the genes involved with meiosis have been disrupted in mice, and some have produced very good models of male infertility. It is likely that at least some of these models may be relevant to human infertility as well.

During prophase and metaphase I of meiosis, sister chromatids are linked together by the synaptonemal complex. *SCP3* forms the lateral elements of the synaptonemal complex, and is necessary for homologous chromosome pairing. When *SCP3* is disrupted, the synaptonemal complex does not form. Male mutant mice undergo spermatogenic arrest during early meiosis, and no post-meiotic germ cells can be identified in the testes [5]. Females are still capable of reproduction, but there is an increase in aneuploidy and embryo death [6].

Another gene that results in abnormal synaptonemal complex formation is *disrupted meiotic cDNA (DMC1)*. *DMC1* was isolated from a screen for meiosis-specific, prophase-induced genes. Disruption of the *DMC1* locus results in meiotic arrest in early prophase 1, with no cells progressing past the zygotene stage, and a malformed synaptonemal complex. Both males and females with a null mutation are infertile [7]. Heat shock protein 70-2 (HSP 70-2) also associates with the synaptonemal complex during meiotic prophase. Synaptonemal complexes in mutant mice assemble and develop through the final pachytene substage, but fail to desynapse, resulting in an absence of normal spermatocytes and an increase in apoptotic cells [8].

The translocated in liposarcoma (*TLS*) gene encodes a RNA binding protein that plays a role as an oncoprotein in the development of human liposarcomas and leukemias. The exact role of *TLS* during meiosis is still unclear, but male mice homozygous for an induced mutation in the *TLS* gene are sterile and have an increase in unpaired and mispaired chromosomes in premeiotic spermatocytes. Chromosomal pairing appears delayed, occurring after the axial elements of the synaptonemal complex have been formed, rather than concurrently, which occurs in the wild type [9].

Several DNA mismatch repair genes have been identified that negatively impact meiosis. *MutS homolog 4* and *5* (*MSH4, MSH5*) are mismatch repair genes expressed predominantly in the testes. Mutant *MSH4* males are infertile and lose the majority of their spermatogonia by adulthood. They exhibit abnormalities in chromosomal pairing at the zygotene stage of prophase 1. Female mutants are subfertile, having a depleted number of oocytes and a decreased ovary size [10]. In *MSH5* mutants, approximately 98 % of chromosomes fail to synapse, and the few chromosomes that do synapse appear to pair incorrectly. Deletion of the *MSH5* locus affects female fertility even more severely, resulting in degeneration of the ovary by adulthood [11].

Two additional mismatch repair genes are *MLH1* and *PMS2*, which form a heterodimer *in vivo*. They also play a role in chromosomal pairing and meiotic crossover events, and mutations in these genes are associated with a predisposition for hereditary non-polyposis colon cancer (HNPCC). Normal chromosomal pairing during meiosis is detected in *MLH1* mice, but post-pachytene stages are rarely observed, with a complete absence of spermatozoa. Females are also infertile [12]. Despite functioning with *MLH1* as a heterodimer, disruption of the *PMS2* locus produces a different phenotype. Chromosomal pairing is disrupted, but spermatids and spermatozoa are observed. The spermatids are round, abnormal and multinucleated, and the spermatozoa have grossly abnormal head shapes with truncated, irregular tails. The epididymal sperm concentration is only 25 % of that in controls. Female fertility is unaffected [13].

Several families of cyclin proteins exist that bind cyclin-dependant-kinases and regulate cell division and growth. *Cyclin A1* is a member of the A-type cyclin family and is expressed exclusively in the germ cell lineages of mice, and in the testis and some myeloid leukemia tumors in humans. *Cyclin A1* is encoded by the *CcnA1* locus, and mice bearing a null mutation for the *CcnA1* gene were sterile due to a block of spermatogenesis before the first meiotic division, while female fertility was normal. Arrest of meiosis was associated with increased germ cell apoptosis and desynapsis abnormalities [14]. Another cyclin, *cyclin D2*, which is activated by FSH and causes female infertility, has also been listed as a male infertility candidate. While male mice with this gene disrupted are still fertile, they do experience a two-to-three-fold decrease in sperm concentration and have a testicular weight approximately 40% of normal [15].

Genes associated with spermatogenic arrest

Several more genes have been identified, in addition to those with a specific role in meiosis, which result in spermatogenic arrest when mutated. *Dazla* is an autosomal homologue to the *DAZ* gene found on the Y chromosome. It is a cytoplasmic factor and anti-*Dazla* antibody stains intensely in pachytene spermatocytes. Male and female knockout mice are sterile, and there is an almost complete absence of germ cells beyond the spermatogonial stage, indicating that arrest takes place prior to the onset of meiosis. Interestingly, though they are still fertile, male mice heterozygous for the mutation have 60% of their sperm visibly abnormal, compared with 15% in control groups. Heterozygotes also have sperm concentrations approximately half of that in the control group population [16].

A gene that has seen a great deal of attention for its role in spermatogenesis is the nuclear transcription factor *cAMP – responsive element modulator* (*CREM*). Through variable splicing, several isoforms of the gene are expressed. Repressor forms of *CREM* are expressed in pre-meiotic germ cells while activator forms are expressed in post-meiotic germ cells. Spermatogenic arrest in *CREM* mutant mice occurs at the round spermatid stage and males are azoospermic. Mice heterozygous for the mutation are subfertile [17, 18].

Genes in the *myb* family encode nuclear proteins that function as regulators of transcription. One of these is *A-myb*, which is expressed primarily in male germ cells and to a lesser degree in the developing mammary tissue of pregnant females. The primary spermatocytes of mutant males degenerate, and there is a complete absence of post-meiotic cells [19]. The *Bcl-2* gene family includes several members that either promote or inhibit apoptosis. *Bcl-w* is an apoptosis-inhibiting member of the family. Targeted disruption of the gene produce healthy mice with azoospermic males; females are unaffected [20]. The *Microchidia* gene (*Morc*) was identified when it was inadvertently disrupted by the insertion of an unrelated transgene, which resulted in complete male sterility and extensive germ cell apoptosis. *Morc* is expressed in a testis-specific fashion in both mice and humans, and spermatogenesis in mice arrests in late prophase 1 [21].

Spermine synthase is an enzyme that converts spermidine to spermine, a highly conserved polyamine important for cell growth and division. A mutation in this gene results in complete spermatogenic arrest and sterility [22]. *TATA box binding protein* (*TBP*) functions as the central core of the general transcription factors that initiate transcription. *TBP-related factor 2* (*TRF2*), is a testis-specific form of *TBP* that is able to take its place in the transcription core. *TRF2* is expressed in the testes of mice and humans from the late pachytene to round spermatid stage. Mice with the *TRF2* gene disrupted are healthy, but azoospermic [23]. *VASA* is a conserved, ATP-dependant RNA helicase with germ cell-specific expression. Mice with the *VASA* gene knocked out appear and develop normally, but males have a depletion of post-meiotic germ cells and are sterile [24].

Proteins associated with the nuclear core

During spermiogenesis approximately 85% of sperm histones are replaced with protamines 1 and 2 (*P1, P2*), assisting in the condensation of the sperm nucleus through the more compact packaging of the sperm DNA [25]. The quantity of *P1* and P2 in the nucleus is approximately equal in fertile controls, but several reports have documented infertile or subfertile populations of men with abnormalities in *P1* and *P2* expression [26]. The obvious importance of the nuclear core proteins in spermatogenesis has led to the investigation of the associated genes.

The transition proteins (*TNP1, TNP2*) are intermediaries that play a role in the conversion from histones to protamines. Mice with a disruption of the *TNP1* gene develop and appear normal, but have increased abnormal sperm head shapes, including an increased number of decondensed heads and severe asthenozoospermia [27]. A knockout of *TNP2* also resulted in highly abnormal morphology of spermatozoa and reduced fertility [28]. A transgenic model that translated *P1* prematurely resulted in precocious nuclear condensation, teratozoo-spermia, and sterile males, some with complete spermatogenic arrest [29]. Another recent report, in which both the *P1* and *P2* loci were separately disrupted, indicates that deletion of either the *P1* or *P2* gene is sufficient to cause infertility [30].

Two additional male infertility candidate gene connected with protamine replacement are *calmodulin dependant protein kinase* (*CamK4*) and *Tarbp2*. *CamK4* is a testis-specific protein kinase that phosphoralates *P2* and is expressed in pachytene spermatocytes. A knockout of the gene resulted in prolonged expression of *TNP* 2, a lack of *P2*, sperm concentrations only 4 % of normal, and complete male infertility [31, 32]. *Tarbp2* is a translational regulator believed to be essential for translation of the protamine mRNAs. A knockout of the gene produced mice with severe oligozoospermia and sterility [33].

Genes related to sperm structure

A number of genes have produced mice with structural sperm abnormalities and partial or complete infertility as a result of their disruption. *EGR4*, a zinc finger transcription factor, is expressed at low levels in the brain and testis. Disruption of the *EGR4* gene results in a partial maturation arrest of germ cells during the pachytene stage, and oligozoospermia. Spermatozoa isolated from *EGR4* mutants show multiple structural abnormalities including separated heads and tails, heads bent back sharply on the tail, and tails that are fragmented, kinked or tightly coiled at the distal end [34]. *JunD* is one of three Jun proteins contributing to the *AP-1* transcription factor complex. A knockout of the *C-jun* or *JunB* proteins is lethal, but *JunD* disruption produces healthy, viable, infertile male mice. Male mutants can be separated into two categories; those with oligo-asthenoteratozoospermia, and ones with normal concentration but decreased motility. During elongation there are severe tail malformations and the microtubules are highly disorganized [35].

THEG is a testis-specific gene with a nuclear signaling domain that is expressed in the round spermatids of mice and humans. Male mutants are infertile and exhibit abnormal sperm elongation and either absent or severely defective tails [36]. *E-MAP-115* is a microtubule-associated protein with high levels of expression in the Sertoli cells and the manchette and flagellum of spermatids. Male mutants were infertile, and exhibited deformed spermatid nuclei and a gradual loss of germ cells. Microtubule associations within the manchette of spermatids and in the Sertoli cells were highly abnormal [37].

Another gene contributing to tail malformations upon mutation is *C-ros*, which is expressed in cells of the epididymis, rather than functioning directly in sperm production. *C-ros* mutants have a high percentage of sperm with decreased motility and bent tails, which are unable to pass through the utero-tubal junction and tangle in a mass at that location. The mass of sperm apparently blocks the junction from being navigated by sperm with normal morphology and motility [38, 39]. *Protein C inhibitor* (*PCI*) is a plasma protein that also has high expression in the male reproductive organs. Disruption of the *PCI* locus results in male, but not female, infertility. The sperm concentration in these mutants is normal, but 95 % of the sperm have abnormal morphology and they are incapable of fertilization with or without the zona pellucida in *in vitro* and *in vivo* studies [40].

Casein kinase II (*CK2*) is a serine-threonine kinase with a couple of different isoforms. The *Csnk2a2* isoform is expressed in the germ cells during the late stages of spermatogenesis. Disruption of the *Csnk2a2* gene produces mice with male infertility, exhibiting oligozoospermia and globozoospermia [41]. The final gene in this category is *Nectin-2*, a cell adhesion molecule that is expressed only during the late stages of spermatogenesis. *Nectin-2* mutant males have a normal concentration of motile sperm, but highly misshapen heads and thin midpieces. Electron microscopy of the sperm reveals that there are mitochondria in the head, the mitochondria in the middle piece are disorganized, the nucleus is condensed but distorted, and the outer dense fibers are jumbled and extended into the head. Male mice mate normally, but are infertile; female fertility is not affected [42].

Genes related to sperm-egg interaction

A small group of genes have been identified that result in male factor defects of fertilization, despite the production of sperm with normal concentration, motility, and morphology, when they are disrupted. Two genes that result in a very similar phenotype are *fertilin* b and *calmegin*. *Fertilin* b is an integrin protein localized to the mammalian sperm plasma membrane. *Calmegin* is a testis-specific, endoplasmic reticulum chaperone protein. When either gene is disrupted, it results in a model with a deficiency in sperm-egg membrane adhesion and fusion *in vitro*, and poor migration of sperm from the uterus through the oviduct and zona binding *in vivo* [43, 44]. Recently it was discovered that *fertilin* b binds specifically to *calmegin*, which likely acts as its chaperone, explaining the common phenotype [45].

One additional gene that shares a similar phenotype to calmegin and fertilin beta upon disruption is *Cyritestin*. Like *fertilin* b, *Cyritestin* is a plasma membrane protein and a member of the ADAM family. *Cyritestin -/-* mice also exhibit an inability to bind and penetrate the zona pellucida, although they fertilize normally when the zona pellucida is removed. Unlike *fertilin* b and *calmegin* knockouts, the sperm from *cyritestin* deficient mice are able to move without difficulty from the uterus through the oviduct [46].

Conclusion

In the course of this review we have attempted to present the genes identified from animal studies that appear to be the most likely candidates for human male infertility. Less than half of the total genes that have been identified in the literature as resulting in male infertility when altered were actually reviewed, since many had other associated complications that were considered too severe to be consistent with human infertility types observed clinically. While several of the genes that have been presented in this review do have some additional characteristics in the mutant males besides infertility, they are generally mild or do not occur until later in life.

As the knowledge gained from animal models is applied in the screening of genetic infertility in humans, it will be important to remember a couple of the lessons learned from the animal studies. First, in almost all cases, heterozygous mutant mice retained fertility, despite severe phenotypes in homozygous mutants. This would imply that single allele mutations alone are not likely to be a cause of infertility in humans. Second, in several cases genes that had expression patterns that were not testis-specific produced infertility phenotypes with few, if any, additional complications.

This suggests that gene redundancy in the somatic tissues can often provide substitutes for missing gene products while spermatogenesis, with fewer redun-dancies, is more vulnerable. Taking this a step further, as we search for candidate infertility genes we should not limit the search to only those genes having testis-specific expression.

Despite advances in diagnoses, a large percentage of male infertility remains unexplained. It has been estimated that 2000 genes regulate the process of spermato-genesis, only 30 of which are located on the Y chromosome [47, 48]. It is highly probable that alterations in some of these genes, including the relative handful presented in this paper may be a cause for some cases of idiopathic male infertility. Determining which of these many genes are clinically relevant will require the close cooperation of both geneticists and clinicians. With the steady advances in molecular biology, including the increased rate at which candidate genes can be identified and evaluated, we are certain to see significant progress in this area of research.

Gene	Phenotype	References
MeiosisRelated		
Cyclin A1	meiotic arrest prior to first division, increased apoptosis	Liu *et al*, 1998
Cyclin D2	decreased sperm concentration	Sicinski *et al*, 1996
DMC1	meiotic arrest at zygotene, abnormal synaptonemal complex	Pittman *et al*, 1998
HSP 70-2	synaptonemal comples fails to desynapse	Dix *et al.* 1997
MLH1	meiotic pachytene arrest	Baker *et al*, 1996

MSH4	abnormal chromosome pairing during zygotene	Kneitz *et al*, 2000
MSH5	incorrect or absent chromosomal pairing	Edelmann *et al*, 1999
PMS2	partial meiotic arrest, oligo- teratozoospermia	Baker *et al*, 1995
SCP3	failure of synaptonemal complex to form	Yuan *et al*, 2000
TLS	delayed, unpaired and mispaired chromosomes	Kuroda *et al*, 2000
Nuclear Core Proteins		
CamK4	Lack of P2, oligozoospermia	Wu *et al*, 2000 andWu and Means, 2000
P1	abnormal spermiogenesis, infertility	Lee *et al*, 1995
P2	same as P1	Cho *et al*, 2000
Tarbp2	oligozoospermia, sterility	Zhong *et al*, 2000
TNP1	oligo- and asthenozoospermia	Yu *et al*, 2000
TNP2	same as TNP1	Adham *et al*, 2001
Spermatogenic Arrest		
A-myb	degeneration of primary spermatocytes	Toscani *et al*, 1997
BCL-w	increased apoptosis	Print *et al*, 1998
Crem	round spermatid stage arrest	Blendy *et al*, 1996 andNantel *et al*, 1996
DAZl	speramatogonial arrest	Ruggiu *et al*, 1997
Morc	increased apoptosis, prophase 1 arrest	Watson *et al*, 1998
Spermine synthase	build up of spermidine, spermatogenic arrest	Lorenz *et al*, 1998
TRF-2	speramatogonial arrest	Zhang *et al*, 2001
Vasa	depletion of post meiotic germ cells	Tanaka *et al*, 2000
Sperm-Egg Interaction		
Calmegin	no sperm-egg adhesion, poor transit through fallopian tubes	Ikawa *et al*, 1997
Cyritestin	sperm fail to bind zona pellucida	Shamsadin *et al*, 1999
Fertilin B	same as calmegin	Cho *et al*, 2000
Stuctural Defects		
CK2	oligo- and globozoospermia	Xu *et al*, 1999
C-ros	bent tails and decreased motility	Yeung *et al*, 1999
EGR4	multiple structural abnormalities	Tourtellotte *et al*, 1999
E-map-115	deformed nuclei, abnormal microtubule associations	Komada *et al*, 2000

JunD	oligo- astheno- teratozoospermia	Thepot *et al*, 2000
nectin-2	misshapen heads and midpieces	Bouchard *et al*, 2000
PCI	abnormal morphology and fertilization	Uhrin *et al*, 1999
THEG	abnormal elongation and tail defects	Yanaka *et al*, 2000

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A single amino acid substitution in *MSH5* results in DNA alkylation tolerance

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Abstract

DNA alkylation tolerance is a major concern in cancer chemotherapy. It has been suggested that mutations in DNA mismatch repair genes may result in alkylation tolerance. This alkylation tolerant phenotype is often manifested in cells lacking an O^6-methylguanine DNA methyltransferase (MTase) activity. However, deletion of each mismatch repair gene in the MTase mutant of a model eukaryotic yeast does not result in alkylation tolerance. We previously isolated an alkylation tolerant mutant and mapped the mutation to *MSH5*. Here we present evidence that a single point mutation that results in a Y823H amino acid substitution, but not deletion, of the *MSH5* gene is responsible for tolerance to killing by DNA alkylating agents. We also find that other preexisting amino acid variations may also enhance alkylation tolerance in the above mutation background. Since *MSH5* encodes a protein homologous to DNA mismatch recognition proteins, mismatch repair genes are frequently mutated in cancers cells and, like mismatch repair genes, *MSH5* is highly conserved from yeast to human, this observation suggests novel mechanisms of chemotherapeutic drug resistance that may occur in certain human cancer patients.

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Keywords: Mismatch repair; Mutagenesis; Yeast

1. Introduction

O^6-Methylguanine DNA methyltransferase (O^6-MeG MTase) activity has been found in all organisms examined to date and is considered primarily responsible for repairing DNA lesions induced by S_N1-type alkylating agents such as *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) and *N*-methyl-*N*-nitrosourea (Pegg, 1990). Mammalian cells lacking O^6-MeG MTase also display enhanced sensitivity to killing by chemotherapeutic bifunctional nitrosoureas such as carmustine, lomustine and streptozotocin (Pegg, 1990; Karran and Hampson, 1996). There have been several independent reports that mutations in mammalian mismatch repair genes such as *hMSH2* (Aquilina et al., 1998), *hMSH6* (Kat et al., 1993; Papadopoulos et al., 1995; Hampson et al., 1997), *hMLH1* (Koi et al., 1994; Hampson et al., 1997) and *hPMS2* (Risinger et al., 1995; Ciotta et al., 1998) may be responsible for rescuing MTase-deficient cells from killing by DNA alkylating agents. Furthermore, at least two mouse mismatch repair-deficient models have been examined with respect to alkylation tolerance (de Wind et al., 1995; Kawate et al., 1998), and a link was established between a mismatch repair defect and alkylation tolerance. An abortive mismatch repair hypothesis was initially proposed by Goldmacher et al. (1986) and then by Branch et al. (1993) to explain the observed phenomena. However, an alternative hypothesis of mismatch repair-induced signaling for apoptosis also gained recent experimental support (D'Atri et al., 1998; Hickman and Samson, 1999).

The DNA mismatch repair system is conserved throughout living organisms. In particular, eukaryotic cells from yeast to human appear to contain a similar set of mismatch repair genes to the extent that each mismatch repair gene in human has a structural and functional homolog in a yeast cell. Our laboratory used *Saccharomyces cerevisiae* as a

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Abbreviations: H-U-H, helix-u-turn-helix; MNNG, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine; MTase, methyltransferase; O^6-MeG, O^6-methylguanine; PCNA, proliferating cell nuclear antigen; SD, synthetic dextrose medium; YIp, yeast integrating plasmid; YPD, yeast extract/peptone/dextrose medium.

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model lower eukaryote and found that deletion of any major mismatch repair genes, including *MSH2*, *MSH3*, *MSH6*, *MLH1* and *PMS1*, in the *mgt1Δ* mutant deficient in MTase activity (Xiao et al., 1991) did not rescue it from killing by MNNG (Xiao et al., 1995). Subsequently, we mutagenized *mgt1Δ* cells, isolated MNNG-tolerant mutants and characterized one of these recessive mutants, XS-14 (Bawa and Xiao, 1997). Transformation of XS-14 cells with each wild-type mismatch repair gene did not alter its alkylation tolerance. Surprisingly, transformation of XS-14 cells with a single-copy *MSH2* homolog *MSH5* can strongly suppress the MNNG-tolerant phenotype, although deletion of the *MSH5* gene did not result in alkylation tolerance. Through genetic analysis, the mutation in XS-14 was mapped within the *MSH5* locus (Bawa and Xiao, 1997). Here we describe our investigation of various *msh5* alleles and demonstrate that a single mutation within the *MSH5* gene that results in an amino acid substitution is responsible for the observed alkylation tolerance.

2. Materials and methods

2.1. Yeast strains and transformation

Yeast strains used in this study are listed in Table 1. XS-803-2C was obtained from Dr. D. Gietz (University of Manitoba, Canada). The creation of its *mgt1Δ::LEU2* and *msh5Δ::hisG* derivatives and the isolation of XS-14 have been described previously (Bawa and Xiao, 1997). Yeast cells were grown in either complete YPD (1% yeast extract, 2% bacto-peptone, 2% glucose) or synthetic SD (0.67% yeast extract without nitrogen bases, 2% glucose) supplemented with amino acids and bases as recommended (Sherman et al., 1983). A lithium-acetate method (Ito et al., 1983) was employed for yeast transformation and other genetic manipulations were performed as previously described (Bawa and Xiao, 1997).

Table 1
S. cerevisiae strains

Strain	Genotype	Source
XS-803-2C	*MATα leu2-3,112 ura3-52 his1-2 hom3-10 can1R*	D. Gietz
WXY1084	XS-803-2C with *mgt1Δ::LEU2*	(Bawa and Xiao, 1997)
XS-14	MNNG-tolerant mutant of WXY1084	(Bawa and Xiao, 1997)
WXY1094	XS14 with *MSH5::URA3*	This study[a]
WXY1086	XS-803-2C with *mgt1Δ::LEU2 msh5Δ::hisG*	(Bawa and Xiao, 1997)
WXY1087	WXY1086 with *msh5-14::URA3*	This study[a]
WXY1088	WXY1086 with *msh5-Y823H::URA3*	This study[a]
WXY1091	WXY1086 with *MSH5::URA3*	This study[a]
WXY1093	WXY1086 with *MSH5-2C::URA3*	This study[a]

[a] See Fig. 2A for the chromosomal structure of the integrants.

2.2. DNA sequencing and sequence analysis

Nucleotide sequences of a 3-kb fragment containing the entire *msh5-14* open reading frame plus a 266-bp promoter region were determined by a dideoxy chain termination method using the Pharmacia T7 DNA sequencing kit. Nested deletions were made from both ends within the *msh5-14* insert and the standard M13 universal and reverse primers were used to determine nucleotide sequences of overlapping fragments. The selected regions of *MSH5-2C* and *MSH5* obtained from Dr. N. Hollingsworth (State University of New York, Stony Brook, NY) were also sequenced. DNA sequence analysis was performed using a DNASTAR program.

2.3. Creation of novel MSH5 alleles

The wild-type *MSH5* gene (*MSH5*) was obtained from Dr. Hollingsworth and cloned into the YIp vector YIplac211 to form YIp-MSH5. Isolation of the *msh5-14* allele using YIp-MSH5 through integration and chromosomal walking has been described (Bawa and Xiao, 1997). In order to create the *MSH5-2C* and *msh5-Y823H* alleles in the same YIp plasmid, a 2.5-kb *Hin*dIII–*Mfe*I fragment containing only the T2467C mutation, and a 2.1-kb *Bam*HI–*Mfe*I fragment containing T-28C, C1299T, A1361G, G1494T, C1570T variations were isolated and used to respectively replace the corresponding region in YIp-MSH5. The resulting replacement was confirmed by sequencing the variable regions. Plasmids YIp-MSH5, YIp-MSH5-2C, YIp-msh5-14 and YIp-msh5-Y823H were linearized with *Sna*BI digestion prior to yeast transformation to create WX1091, WXY1093, WXY1087 and WXY1088, respectively. Several independent transformants from each transformation were examined, from which a representative strain was saved for further studies. To create WXY1094, plasmid YIp-MSH5 was linearized and used to transform XS-14 so that the plasmid vector sequence in the resulting integrants is flanked by *msh5-14* and *MSH5* alleles at the *MSH5* locus.

2.4. Alkylation sensitivity tests

Two methods for quantitating cell killing were used. For liquid killing, freshly grown cells at a concentration of 2×10^7 cells/ml were treated with 40 μg/ml MNNG for various times, washed, diluted, plated on YPD and incubated at 30 °C. For gradient plate assays, a gradient was created by pouring 30 ml of SD+MNNG medium into a tilted, square Petri dish. After solidification, the plate was placed level and overlaid with 30 ml of SD medium. Overnight cultures were printed across the gradient using microscope slides and the plates were incubated for 3 days. The length of growth across the gradient was measured and relative growth was expressed as a percentage of full-length growth on the plate without MNNG.

3. Results

3.1. *msh5-14* contains a single point mutation

In order to understand the mechanism by which a point mutation in *MSH5* results in alkylation tolerance, we isolated the mutated *MSH5* allele, *msh5-14*, from XS-14 and determined the entire nucleotide sequence of the *msh5-14* open reading frame plus a 266-bp promoter region and compared it with the published *MSH5* sequence (Hollingsworth et al., 1995). To our surprise, as many as six nucleotide sequence variations (T-28C, C1299T, A1361G, G1494T, C1570T and T2467C, relative to the *MSH5* translation start) were observed, four of which result in amino acid substitutions (Q454R, L498F, L524F and Y823H). These multiple mutations were either due to some previously existing variations in the parental strain (XS-803-2C) used in this study or due to a consequence of excessive mutagenesis treatment in the *mgt1Δ* mutant background, which is known to be highly mutagenic (Xiao et al., 1991). To address the above possibilities, we determined the entire *MSH5* allele (*MSH5-2C*) from the parental strain XS-803-2C. This allele contains all the above nucleotide substitutions except T2467C (Fig. 1, results for other altered nucleotide sequences are not shown), indicating that a single mutation corresponding to Y823H is responsible for the observed alkylation tolerance.

3.2. *Y823H* substitution results in alkylation tolerance

The DNA sequencing result appears to support the notion that a single amino acid substitution (Y823H) in Msh5 results in alkylation tolerance. However, one cannot rule out the possibility that other preexisting amino acid sequence variations also contribute to the observed phenotype. In order to address this possibility, we made a series of constructs in a yeast YIp integration vector (Fig. 2A) containing *MSH5*, *MSH5-2C*, *msh5-14* or *msh5-Y823H*. These plasmids were used to transform a *mgt1Δ msh5Δ* double-mutant strain so that the *MSH5* allele was integrated at the *msh5Δ* locus under the control of its native promoter. As summarized in Fig. 2B from a gradient plate assay, both *msh5-14* and *msh5-Y823H* were able to rescue the severe sensitivity of *mgt1Δ msh5Δ* host cells from killing by MNNG, whereas integration of *MSH5* or *MSH5-2C* failed to alter the alkylation sensitivity of the *mgt1Δ msh5Δ* mutant. However, the level of MNNG tolerance in *msh5-*

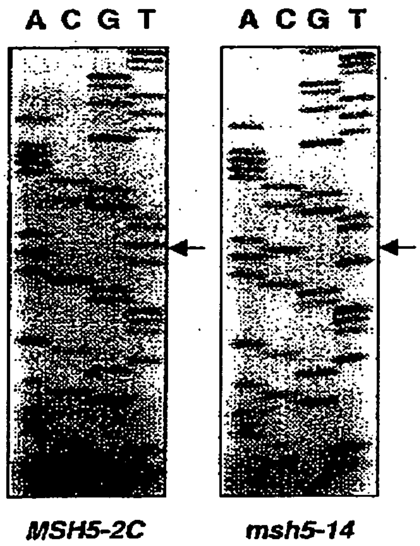

Fig. 1. Nucleotide sequences of *MSH5-2C* and *msh5-14* around the helix-turn-helix coding region. Arrows point to the T-to-C transition in *msh5-14*.

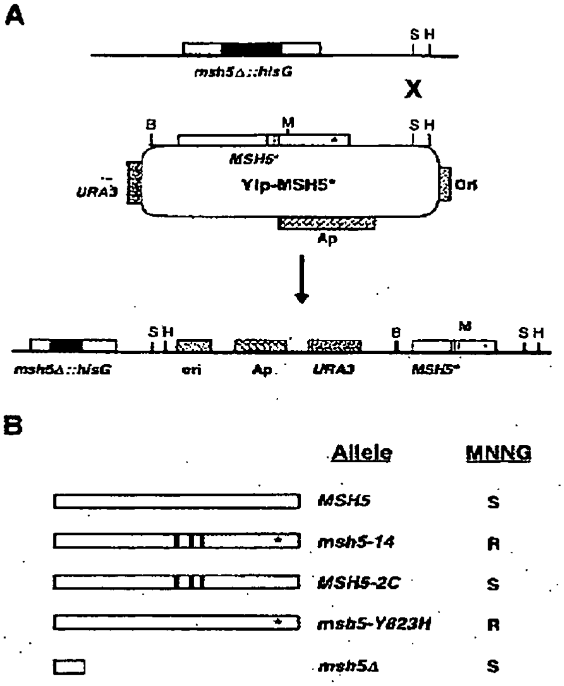

Fig. 2. Alkylation sensitivity of different *MSH5* alleles. (A) Source of *MSH5* alleles and their integrant structure. YIp plasmids carrying various *MSH5* alleles (*MSH5**) were created in vitro as described in Materials and methods. These plasmids were linearized at the unique *Sna*BI site prior to yeast transformation, resulting in site-specific integration and the chromosomal structure at the *MSH5* locus as shown. The *msh5Δ::hisG* allele contains an inactive *MSH5* gene deletion and replacement with *hisG*, which is an *msh5* null allele. Open box represents the *MSH5* open reading frame. Three vertical bars represent nucleotide variations that results in three amino acid substitutions, Q454R, L498F and L524F, and the asterisk indicates the T2467C mutation that results in Y823H. Restriction sites: B, *Bam*HI; H, *Hin*dIII, M, *Mfe*I; and S, *Sna*BI. (B) Summary of MNNG killing experiments from a gradient plate assay. WXY1086 is *mgt1Δ* and carries an *msh5Δ::hisG* allele, which was used as the parental strain. Other strains were integrants of various *MSH5* alleles into WXY1086. S, MNNG-sensitive; R, MNNG-tolerant.

14 and *msh5-Y823H* integrants were apparently different. To further quantitatively determine the level of alkylation sensitivity, the *msh5-14* and *msh5-Y823H* integrants were compared with their isogenic wild-type and parental *mgt1Δ msh5Δ* double mutant in a liquid killing experiment. While *mgt1Δ msh5-14* is up to 10 times more resistant to MNNG than the *MGT1* wild-type cells, *mgt1Δ msh5-Y823H* is about 10 times more sensitive to MNNG than the wild-type cells (Fig. 3), suggesting that at least one of the other three amino acid substitutions (Q454R, L498F or L524F) contributes to the observed alkylation tolerance in XS-14. However, these three amino acid substitutions alone do not appear to result in an altered phenotype in alkylation tolerance, since the levels of MNNG-induced killing in *mgt1Δ msh5Δ*, *mgt1Δ MSH5* and *mgt1Δ MSH5-2C* are indistinguishable (Fig. 2B and data not shown). Nevertheless, cells carrying the *msh5-Y823H* allele are two to three orders of magnitude more resistant to MNNG than their isogenic *msh5Δ* cells (Fig. 3).

3.3. *MSH5* and *msh5-14* are co-dominant

Previously, we observed that transformation of XS-14 with a single-copy *MSH5* significantly reduced the MNNG tolerance but did not revert XS-14 to its parental level of extreme MNNG sensitivity (Bawa and Xiao, 1997). To distinguish whether this was due to co-dominance of *MSH5* and *msh5-14*, or unknown mutations in XS-14 outside of the *MSH5* gene, we isolated the *msh5-14* allele and integrated it into a wild-type *MSH5* strain. Cells carrying both alleles in the same chromosome locus display an intermediate level of MNNG-tolerance (Fig. 4) comparable to the previous plasmid transformation experiment (Bawa and Xiao, 1997), suggesting that *msh5-14* is indeed co-dominant with *MSH5*.

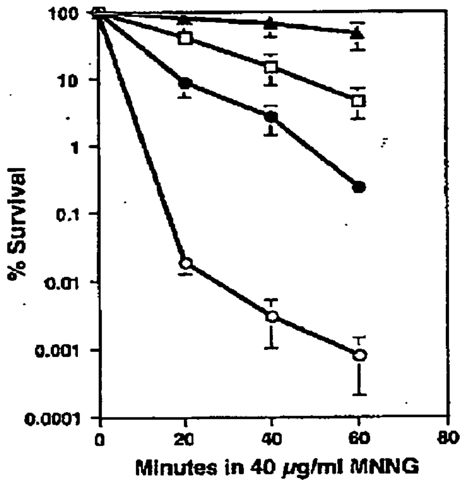

Fig. 3. Levels of alkylation sensitivity of *msh5-14* and *msh5-Y823H* integrants compared with isogenic wild-type and the parental *mgt1Δ msh5Δ* mutant. An MNNG-induced liquid killing experiment was performed with (□) XS-803-2C (wild-type); (○) WXY1086 (*mgt1Δ::LEU2 msh5Δ::hisG*); (●) WXY1088 (*mgt1Δ::LEU2 msh5-Y823H::URA3*); and (▲) WXY1087 (*mgt1Δ::LEU2 msh5-14::URA3*). All strains are isogenic to XS-803-2C. The results are presented as the average of three independent experiments with standard deviations shown as error bars.

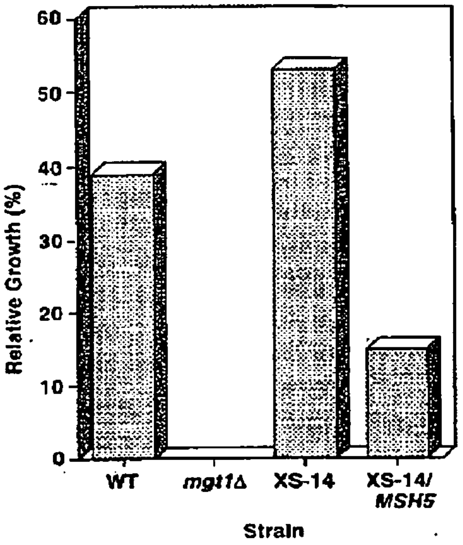

Fig. 4. The *msh5-14* mutation is co-dominant with *MSH5* over alkylation tolerance. Results of a quantitative gradient plate assay are presented as relative growth on a 10 μg/ml MNNG plate. The gradient plates were incubated at 30 °C for 72 h before recording the data.

4. Discussion

We report here the detailed characterization of the *msh5-14* mutation that results in an increased alkylation tolerance. We found a single de novo mutation in the *msh5-14* allele along with several preexisting sequence variations. We demonstrated that the resulting Y823H single amino acid substitution is sufficient to cause alkylation tolerant phenotypes. It is interesting to note that the Y823H substitution falls in a well-conserved region known as a helix-u-turn-helix (H-U-H) motif (Hsieh, 2001). This motif is located in the Taq MutS dimer interface (Biswas et al., 2001), and has been proposed to play a key rôle in modulating ATP hydrolysis and ATP-mediated mismatch recognition by Msh2 and Msh6 (Alani et al., 1997). More intriguingly, the Y823H substitution in Msh5 makes this residue identical to that of Msh2 and Msh6 (Eisen, 1998) (see Fig. 5). Hence, it is conceivable that a Y823H substitution does not abolish its H-U-H function. Instead, it may be a gain-of-function mutation. At least three lines of evidence support the notion that *msh5-14* is not a simple recessive mutation. Firstly, *mgt1Δ* cells carrying both *MSH5* and *msh5-14* alleles display an intermediate level of MNNG tolerance, showing that *msh5-14* is a co-dominant allele. Secondly, *msh5-14* is

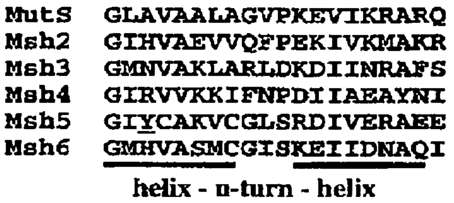
MutS GLAVAALAGVPKEVIKRARQ
Msh2 GIHVAEVVQFPEKIVKMAKR
Msh3 GMNVAKLARLDKDIINRAFS
Msh4 GIRVVKKIFNPDIIAEAYNI
Msh5 GIYCAKVCGLSRDIVERAEE
Msh6 GMHVASMCGISKEIIDNAQI
helix - u-turn - helix

Fig. 5. Amino acid sequence alignment of *E. coli* MutS and *S. cerevisiae* MutS homologs at the H-U-H region. The 823Y residue of Msh5 is underlined. Two horizontal bars represent the predicted α-helices.

not a simple loss-of-function mutation, since deletion of *MSH5* did not result in alkylation tolerance. Finally, a diploid heterozygous for *MSH5/msh5-14* is sterile even in the presence of the wild-type *MSH5* (Bawa and Xiao, 1997), suggesting that *msh5-14* is dominant over *MSH5* with respect to meiotic phenotypes. Since the H-U-H domain is possibly involved in heterodimerization, we tested by a yeast two-hybrid analysis whether Msh5(Y823H) alters its interaction with other mismatch repair proteins and PCNA. Our preliminary results to date (not shown) do not appear to lend support to this hypothesis. However, it remains possible that Msh5 may back up some Msh2/Msh6 functions, especially in mismatch recognition. Alternatively, Msh5-14 may compete with Msh2/Msh6 for MMR complex formation. Additional experiments are needed to address these possibilities.

In summary, our discovery that a single amino acid substitution in Msh5 results in alkylation tolerance provides a new direction to investigate this clinically and fundamentally important issue. Several important implications can be drawn from this study. Firstly, the *MSH5* gene has previously been described to play a role only in meiotic cells in both yeast (Hollingsworth et al., 1995) and mammals (Edelmann et al., 1999). However, the phenotypes we observed with the point mutation and natural variations suggest that *MSH5* plays a role in mitotic cells. Secondly, the point mutation we identified in *MSH5* suggests that *MSH5* may indeed be involved in alkylation tolerance. However, the fact that deletion of *MSH5* or any other mismatch repair genes did not result in alkylation tolerance indicates that the underlying mechanism is probably far more complicated than that predicted by a simple abortive mismatch repair hypothesis. Thirdly, the fact that *msh5-14* (or *msh5-Y823H*) is co-dominant over *MSH5* in alkylation tolerance and is dominant over *MSH5* in meiosis (spore viability) indicates that, if the same is true in human cells, this type of mutation will not penetrate germ cells and will remain a rare somatic mutation. Fourthly, we also observe strain variations within the *MSH5* gene: up to five nucleotide substitutions and three amino acid substitutions. This is clearly higher than reported genomic variations in laboratory yeast strains, but may not be unique to the *MSH5* gene. Interestingly, although these variations per se do not result in alkylation tolerance, they (or one of them) appear to enhance the *msh5-Y823H* mutation phenotype with respect to alkylation tolerance. This phenomenon is reminiscent of genomic sequence diversity within the human population and susceptibility factors to cancer. If similar variations are found in human Msh5, they may be considered as predisposition factors. Finally, mismatch repair defects have been linked not only to tolerance to experimental alkylating agents and therapeutic chloroethylating agents (Karran and Hampson, 1996), but also to other DNA-damaging agents such as cisplatin (Drummond et al., 1996). Hence, our results may provide an alternative mechanism of alkylation tolerance in mammalian cells and impact future clinical diagnosis and treatment of cancer patients. Clearly, this study needs to be further advanced to address some of the above questions.

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